



Chemical-agnostic hazard prediction: Statistical inference of *in vitro* toxicity pathways from proteomics responses to chemical mixtures



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ABSTRACT

Toxicity pathways have been defined as normal cellular pathways that, when sufficiently perturbed as a consequence of chemical exposure, lead to an adverse outcome. If an exposure alters one or more normal biological pathways to an extent that leads to an adverse toxicity outcome, a significant correlation must exist between the exposure, the extent of pathway alteration, and the degree of adverse outcome. Biological pathways are regulated at multiple levels, including transcriptional, post-transcriptional, post-translational, and targeted degradation, each of which can affect the levels and extents of modification of proteins involved in the pathways. Significant alterations of toxicity pathways resulting from changes in regulation at any of these levels therefore are likely to be detectable as alterations in the proteome. We hypothesize that significant correlations between exposures, adverse outcomes, and changes in the proteome have the potential to identify putative toxicity pathways, facilitating selection of candidate targets for high throughput screening, even in the absence of *a priori* knowledge of either the specific pathways involved or the specific agents inducing the pathway alterations. We explored this hypothesis *in vitro* in BEAS-2B human airway epithelial cells exposed to different concentrations of Ni^{2+} , Cd^{2+} , and Cr^{6+} , alone and in defined mixtures. Levels and phosphorylation status of a variety of signaling pathway proteins and cytokines were measured after 48 h exposure, together with cytotoxicity. Least Absolute Shrinkage and Selection Operator (LASSO) multiple regression was used to identify a subset of these proteins that constitute a putative toxicity pathway capable of predicting cytotoxicity. The putative toxicity pathway for cytotoxicity of these metals and metal mixtures identified by LASSO is composed of phospho-RPS6KB1, phospho-p53, cleaved CASP3, phospho-MAPK8, IL-10, and Hif-1 α . As this approach does not depend on knowledge of the chemical composition of the mixtures, it may be generally useful for identifying sets of proteins predictive of adverse effects for a variety of mixtures, including complex environmental mixtures of unknown composition.

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Introduction

The National Academy of Sciences has proposed approaching the assessment of toxicological risk of chemical exposures from the standpoint of toxicity pathways, which are defined as normal biological pathways that, if sufficiently perturbed, lead to possible adverse health outcomes [1]. The premise is that if such pathways can be identified, then the risk posed by toxicant exposure may be inferred from the extent of pathway disruption. The appeal of this approach is that it focuses primarily on the extent of pathway perturbation, and thus is largely independent of the specific chemical or chemical mixture exposure inducing the pathway alteration.

Accordingly, the approach should be applicable to assessing the toxicity of chemical mixture exposures as well as single agent exposures. Once a toxicity pathway is identified, measurement of alteration of a relatively small number of key events within that pathway in response to toxicant exposures can provide a rapid means of predicting the extent of toxicity that the exposure will induce. A key conceptual difficulty presented by this approach for predictive screening is the identification of appropriate pathways and key events to measure.

An inherent requirement for the application of toxicity pathway key event monitoring to predict an adverse outcome is that there must exist robust relationships between the pathway disrupting exposures, the extent of alteration of the key events being monitored, and the adverse outcome. If a node in a toxicity pathway is relatively unaffected by the exposure, or if alterations of that node are not strongly linked to the adverse outcome, then

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assessment of the status of that node in response to an exposure is not likely to yield a useful prediction of the adverse outcome induced by that exposure. Therefore, for an altered pathway node to be a useful indicator of toxicity pathway alteration, the extent of its alteration will exhibit significant correlations with both exposure and adverse outcome. In addition, the magnitude of node alteration in response to the exposure ideally should be large enough at the time of measurement that the measured change in the node can be accurately assessed experimentally.

Alteration of biological pathways in response to chemical exposures can occur at many different levels, including large scale chromatin reorganization, induction of mutations in pathway genes, modulation of transcription, post-transcriptional regulation of translation, post-translational protein modifications, and targeted degradation of pathway proteins. Each of these levels of regulation has the potential of affecting the levels and extents of modification of pathway proteins, and thus pathway function, in response to pathway perturbing exposures. Alterations at the level of the proteome reflect the cumulative result of regulation at all of these levels, and are therefore more likely to be reflective of pathway dysregulation and predictive of adverse health effects than alterations at upstream levels.

These considerations suggest that, at a practical level, it should be possible to identify key nodes in putative toxicity pathways by a relatively straightforward assessment of the correlations between a series of exposures, a series of measurements of cellular molecular events, and the extent of induction of an adverse effect elicited in cells by the exposures, even in the absence of detailed *a priori* knowledge of the toxicity pathways involved. We have examined this approach capitalizing on the availability of a previously published data set [2] generated by *in vitro* exposure of BEAS-2B human airway epithelial cells to different concentrations of Cd^{2+} , Cr^{6+} , and Ni^{2+} , both individually and in combinations at different concentrations, and then assessing the levels of induced cytotoxicity as well as levels of selected proteins and extent of post-translational protein modifications in the cells. The proteins assessed were selected based upon a literature survey of the effects of the metals on cells, including induction of reactive oxygen stress, inflammation, and altered cell signaling. Multiple regression analysis was performed to identify a parsimonious subset of altered proteins for which the extent of alteration is predictive of the extent of cytotoxicity among the mixtures examined.

Materials and methods

Cell treatments

Details of the cell treatments, determinations of protein level and extent of modification, and cytotoxicity have been explained in a previous publication [2]. Briefly, BEAS-2B human respiratory epithelial cells (American Type Culture Collection, Rockville, MD) were cultured in LHC-9 medium. The cells were maintained at $37 \pm 1^\circ\text{C}$, $85 \pm 5\%$ relative humidity, and $5 \pm 1\%$ CO_2 . Medium was replaced every second day and cells were sub-cultured at approximately 80% confluence by washing with HEPES-buffered saline solution, followed by a wash with Versene and dislodged with TrypLe. For cytotoxicity measurements, cells were seeded at an initial

density of 30,000 cells/ cm^2 in 0.125 ml of media per well of a 96-well plate. For proteomic measurements, cells were seeded at 40,000 cells/ cm^2 in 150 cm^2 flask.

Treatment with metals and cytotoxicity assay

Forty eight hours after seeding, 0.125 ml of media containing the metal treatment was applied to each well of the cytotoxicity assay plate. The metal treatment conditions used are shown in Table 1. The flasks for the proteomic endpoints were treated at the same time as the cytotoxicity plates. The culture medium was removed and replaced with 15 ml of LHC-9 and 15 ml of the appropriate metal dilution. All exposures to the metal treatments were forty eight hours for both cytotoxicity and proteomic endpoints.

Cytotoxicity of the metals in BEAS-2B cells was determined by the neutral red assay. Briefly, medium was removed and sterile replaced with LHC-9 medium containing 0.003% neutral red dye. The cells were incubated for 3 h at 37°C . Neutral red medium was removed, wells were rinsed with Dulbecco's phosphate buffered saline, and 100 μl of extraction buffer (50% ethanol, 1% acetic acid in water) was added to each well. Plates were gently shaken for 20–40 min and absorbance measured at 540 nm to measure dye uptake. Each plate included cell-free blank wells and untreated control cell wells.

Protein symbols

Proteins are referenced by their HGNC approved symbols. In our previous publication [2], MAPK8, mitogen-activated protein kinase 8, was referred to as JNK, and RPS6KB1, ribosomal protein S6 kinase B1, was referred to as p70S6K.

Protein assays

Assessment of levels of expression or post-translational modifications of 21 proteins across 29 different treatments varying in the amounts of Ni^{2+} , Cd^{2+} , or Cr^{6+} present was carried out using MSD® Multi-Spot immunoassays with electrochemiluminescent detection (Meso Scale Discovery, Inc., Rockville, MD) as previously described [2]. Endpoints measured were total protein for Hif-1 α , p53, GM-CSF, IFN- γ , IL-1 β , IL-10, IL-12p70, IL-2, IL-6, IL-8, and TNF- α , as well as phospho-p53 (Ser15), phospho-GSK-3 β (Ser9), phospho-RPS6KB1 (Thr421/Ser424), phospho-Akt (Ser474), phospho-EGFR (Tyr1173), phospho-MAPK8 (Thr183/Tyr185), phospho-ERK1/2 (Thr202/Tyr204; Thr185/Tyr187), phospho-ErbB2 (Tyr1248), cleaved PARP (Asp214), and cleaved CASP3 (Asp 175). Assays were conducted according to manufacturer's protocols. Results from each assay were expressed as mean fold change relative to levels in untreated control cell cultures.

Statistical analyses

Statistical analyses were performed using SigmaPlot for Windows version 12.5 (Systat Software, Inc., San Jose, CA), and SAS version 9.4 (SAS Institute, Cary, NC). Feature selection by multiple regression for modeling cytotoxicity as a function of protein fold-changes was performed with R version 3.2.2 (<http://>

Table 1

Metal treatments. Each column in the table represents a tested treatment condition containing the indicated.

Metal	Concentration (μM)																												
Ni ²⁺	0	30	60	75	100	0	30	60	0	30	60	0	30	60	0	30	60	0	30	60	0	30	60	0	30	60	0	30	60
Cd ²⁺	0	0	0	0	0	5.6	5.6	5.6	7.5	7.5	7.5	0	0	0	5.6	5.6	5.6	7.5	7.5	7.5	0	0	0	5.6	5.6	5.6	7.5	7.5	7.5
Cr ⁶⁺	0	0	0	0	0	0	0	0	0	0	0	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	1.8	1.8	1.8	1.8	1.8	1.8	1.8	1.8

μmolar concentrations of each metal.

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